The role of Kainate receptor auxiliary subunits NETO1 and NETO2 in development of hippocampal circuitry

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Original publications

This dissertation is based on the original publications listed below. In addition, some unpublished data are presented in the thesis.

I **Orav E**, Atanasova M, Shintyapina A, Kesaf S, Kokko M, Partanen J, Taira T, Lauri SE. NETO1 guides development of glutamatergic connectivity in the hippocampus by regulating axonal kainate receptors. *eNeuro*. 2017 Jul 3;4(3). pii: ENEURO.0048-17.2017. doi: 10.1523/ENEURO.0048-17.2017 (2017)

II **Orav E**, Dowavic I, Huupponen J, Taira T, Lauri SE. NETO1 regulates postsynaptic Kainate Receptors in CA3 interneurons during circuit maturation. *Mol Neurobiol*. 2019 May 1. doi: 10.1007/s12035-019-1612-4. (2019)

Author's contribution to the studies included in the thesis:

Article I: Doctoral candidate Ester Orav (EO) participated in experimental design and performed most of the experiments included in the article. EO contributed to the reverse transcriptase (RT) - PCR experiments and cloning of the viral vectors. EO conducted all neuron culture experiments including dissection, preparation and maintenance of the primary neuron cultures, viral infections, confocal imaging and image analysis. In addition, EO performed most of the single-cell electrophysiology experiments on acute and cultured organotypic slices and analyzed the collected data. EO prepared all the figures and contributed to writing of the manuscript.

Article II: EO designed and performed most of the experiments in the article, including all neuron culture experiments, lentiviral infections, confocal imaging and image analysis. EO performed most of the electrophysiological recordings from CA3 interneurons and analyzed the data. EO prepared all the figures and wrote the manuscript together with Sari Lauri.

Unpublished data: EO produced all the unpublished data included in the thesis.

Abbreviations

ACSF	artificial cerebrospinal fluid
AMPA(R)	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (receptor)
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CA1-3	cornu ammonis 1-2, sub regions in hippocampus
cDNA	complementary DNA
CNS	central nervous system
C-terminus	carboxy terminus
CUB domain	complement C1r/C1s, Uegf, Bmp1 domain
DG	dentate gyrus
DIV	days in vitro
EC	enthorinal cortex
EPSC	excitatory postsynaptic current
GABA	γ-aminobutyric acid
GAD67	glutamate decarboxylase 67 kDa isoform
GDP	giant depolarizing potential
$G_{i/o}$ protein	guanine nucleotide-binding inhibitory/other protein
GluA2/3	AMPA-type glutamate receptor subunits 2/3
GluK1-5	kainate-type glutamate receptor subunits 1-5
HA-tag	human influenza hemagglutinin tag, YPYDVPDYA
KA(R)	kainate (receptor)
KO	knock-out
LDLa domain	low-density lipoprotein receptor class A domain
LTD	long-term depression
LTP	long-term potentiation
M1-M4	KAR membrane spanning domains 1-4
I_{mAHP}	medium afterhyperpolarizing current
MAP2	microtubule associated protein 2
MEA	microelectrode array
mEPSC	spontaneous action potential independent miniature excitatory postsynaptic current
MF	mossy fiber
mM	millimolar
mRNA	messenger RNA
NETO	neuropilin and tolloid-like
NMDA(R)	N-methyl-D-aspartate (receptor)
N-terminus	amino-terminus
P(day)	postnatal day
PDZ	acronym from PSD95/synapse associated protein 90 (SAP90), Drosophila discs large homolog 1 (Dlg1), and zonula occludens protein 1 (ZO-1)
PKC	protein kinase C
PSD95	postsynaptic density 95

Q/R editing	exchange of glutamine (Q) to arginine (R)
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcriptase PCR
I_{sAHP}	slow afterhyperpolarizing current
SEM	standard error mean
sEPSC	spontaneous excitatory postsynaptic current
sIPSC	spontaneous inhibitory postsynaptic current
Syn	Synaptophysin
TA-cloning	thymine(T) and adenine(A) based cloning
t-KAR	tonically active kainate receptor
TrkB receptor	tyrosine receptor kinase B; BDNF/NT-3 receptor
WT	wild-type

Abstract

Neural circuits emerge when neurons become connected by synaptic contacts. In rodents, this process begins already before birth and continues during the first postnatal weeks. The initial steps of synapse formation are guided by intrinsic molecular cues. The emerging synaptic contacts are then refined and fine-tuned by activity-dependent mechanisms.

Kainate-type glutamate receptors (KARs) are involved in synapse formation and refinement during this “critical period” by regulating neurotransmitter release and neuronal excitability in both principal cells and interneurons in a developmentally restricted manner. Functional KAR tetramers are assembled from various combinations of five core subunits GluK1-5 and supplemented with auxiliary subunits, Neuropilin and tolloid-like proteins (NETO) 1 and 2 that are not part of the pore-forming receptor. KAR interaction with NETOs affects multiple aspects of KARs like subcellular localization of the receptor complex, receptor gating and current kinetics, and even KAR affinity to main agonists, kainate and glutamate.

Despite the accumulating evidence emphasizing the functional significance of NETOs in regulating KAR functions in the adult brain, the role of NETO/KAR complex in the immature brain remains elusive. The main aim of this study was to clarify the physiological significance of NETO/KAR complex in the maturation of hippocampal circuitry. First, we found that NETO1 is an important regulator of physiologically relevant KAR activity at immature glutamatergic synapses. NETO1 deficiency significantly reduced axonal delivery of KARs resulting in loss of presynaptic KAR function and delayed maturation of CA3-CA1 synapses. At the network level, NETO1 deficiency caused impaired synchronization between areas CA3 and CA1 of the hippocampus. This phenotype was fully rescued by GluK1c expression at CA3 principal neurons, emphasizing the role of NETO1 and axonal GluK1-containing immature-type KARs in the development of CA3-CA1 synapses.

Next, we showed that NETO1 is necessary for the dendritic delivery of KAR subunits and for formation of KAR-containing synapses in cultured GABAergic neurons. In CA3 interneurons, loss of NETO1 disrupted postsynaptic and metabotropic KAR signaling, while a subpopulation of ionotropic KARs in the somatodendritic compartment remained functional. NETO1 was not necessary to maintain the excitability of the immature CA3 network at physiological levels. However, kainate-dependent modulation of network bursts and GABAergic transmission in the developing hippocampus was significantly impaired in the absence of NETO1.

In conclusion, these new findings elucidate the cellular mechanisms and physiological significance of NETO/KAR interaction in hippocampal principal cells and interneurons during the first week of postnatal development. This early period of neural network development is extremely sensitive to external stimuli. Accordingly, disturbances in circuit structure or activity patterns that take place during this “critical period” could predispose to neuropsychiatric disorders later in life.

1. REVIEW OF THE LITERATURE

1.1 Introduction

Development of neuronal circuits is a dynamic process that involves a rapid and simultaneous formation and elimination of synaptic connections. According to the emerging view the immature-type synapses support rapid morphological plasticity that is optimally driven by the endogenous activity patterns of the developing networks. After the "critical period" of development synaptic transmission and plasticity becomes more controlled, suitable for their functions within the mature network. The same plasticity mechanisms that allow the developing networks to be fine-tuned to their adult functions also make them vulnerable to external disturbance. It is well known that early adverse experiences, involving unusually strong activation of the limbic system, heighten susceptibility to neuropsychological problems later in life. Thus, comprehensive knowledge of the mechanisms guiding network assembly is not only important for understanding the physiological functions of a healthy brain but also critical for providing insight into pathogenesis of developmentally originating neurological diseases.

KARs make excellent candidates for this area of research due to developmental regulation of expression and unique functional properties of these receptors in the limbic system, especially in the hippocampus. KARs mediate spatially and temporally restricted signaling that may powerfully regulate excitability and morphogenesis of the developing network and consequently, guide the proper wiring of the brain (reviewed by Hanse et al., 2009; Lauri and Taira, 2011, 2012). Furthermore, KARs have been linked to several nervous system pathologies such as anxiety, epilepsy and mood disorders (reviewed by Pinheiro and Mulle, 2006; Lerma and Marquez, 2013). This calls for a detailed understanding of the molecular and cellular mechanisms by which KARs regulate the development and maturation of neuronal networks.

In the adult hippocampus, various physiological properties of KARs are regulated by auxiliary subunits NETO1 and 2. For instance, NETOs have been shown to affect current kinetics, agonist affinity, and synaptic delivery of KARs in a subunit, synapse and cell type dependent manner (reviewed by Copits and Swanson, 2012). Defining the roles of NETO auxiliary subunits is required to fully understand the unique developmentally regulated actions of KARs. This might resolve critical poorly understood aspects related to KAR functions in normal and pathological circuit development.

1.2 Overview of KARs

Ionotropic glutamate receptors play a critical part in mediating and modulating excitatory synaptic transmission in the brain. Kainate receptors belong to a glutamatergic ligand gated ion channel family that also include widely studied AMPA and NMDA receptors. KARs exhibit a characteristic that has not been classically associated with ionotropic receptor function. Namely, in addition to the ionotropic function, KARs signal through non-conventional metabotropic mechanism that involves G-protein activation (reviewed by Contractor et al., 2011).

KARs comprise of five different subunits GluK1-5 (Figure 1), that can be assembled in different combinations to form a functional tetrameric receptor. KAR subunits can be grouped into low-affinity (GluK1-3) and high-affinity (GluK4-5) subtypes based on their affinity for glutamate (Figure 1). Low-affinity subunits GluK1-3 can form functional membrane expressed homomers or heteromers. High-affinity subunits GluK4 and GluK5 can only be incorporated into heteromeric KARs that include obligatory GluK1-3 subunits (reviewed by Contractor et al., 2011; Carta et al., 2014). Additional KAR subunit variety arises from alternative splicing of GluK1-3 and Q/R editing of GluK1 and GluK2 pre-mRNA (Figure 1) (reviewed by Contractor et al., 2011; Swanson et al., 1996).

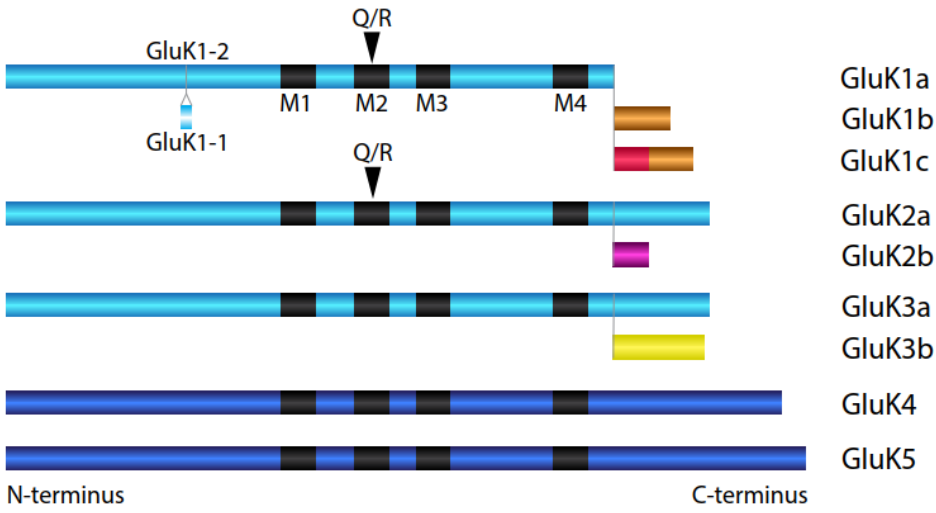


Figure 1. KAR subunits GluK1-5 and their splice variants.

Low-affinity subunits GluK1-GluK3 are depicted in light blue and are required for the formation of heteromeric KARs. High-affinity subunits GluK4-GluK5 are shown in dark blue. All subunits contain N-terminal domain, four membrane spanning domains M1-M4 (black) and intracellular C-terminal domain. M2 domain in GluK1 and GluK2 subunits contains a Q/R editing site that determines Ca^{2+} permeability of the tetrameric receptor. M1, M3 and M4 domains are transmembrane, while M2 is a reentrant poor loop. GluK1 subunit has two N-terminal splice variants (GluK1-1 and GluK1-2) and three C-terminal domains (GluK1a, GluK1b, GluK1c). Both GluK2 and 3 have two splice variants GluK2a and b, GluK3a and b. (Adapted from Contractor et al., 2011).

NETO1 and NETO2 have emerged as auxiliary subunits of native KARs. NETOs are not incorporated into KAR pore-forming tetramer. Instead, they directly bind KARs via extracellular N-terminal domain and maintain the interaction throughout KAR life-time (Figure 2) (reviewed by Lerma, 2011; Copits and Swanson, 2012). NETO interaction provides an additional regulation mechanism and has numerous implications to KAR properties that will be discussed in the later chapters.

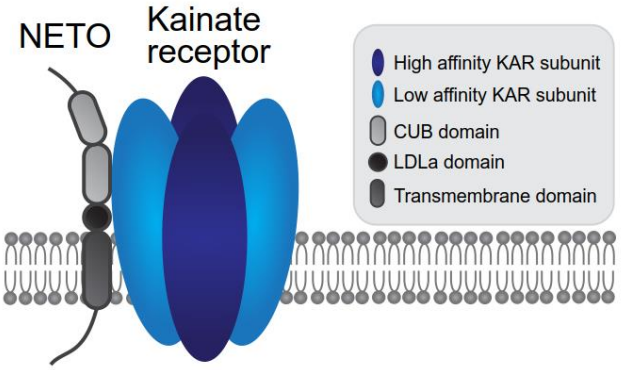


Figure 2. Schematic representation of NETO/KAR complex.

Auxiliary proteins NETO1 and NETO2 (depicted as NETO) interact with tetrameric KAR via extracellular domains. The second CUB domain of NETO mediates the interaction with KARs. (Modified from Lerma, 2011).

Subunit composition ultimately determines the subcellular localization, signaling mechanisms and functions of KARs. Presynaptically localized KARs typically modulate neurotransmitter release while somatodendritic KARs participate in modulating synaptic transmission and neuronal excitability either by ionotropic or metabotropic signaling mechanisms (reviewed by Lerma and Marquez et al., 2013). For instance, postsynaptic KARs at Mossy Fiber (MF)-CA3 synapses in hippocampus (Figure 3) elicit a characteristically slow postsynaptic current (Castillo et al., 1997; Vignes and Collingridge, 1997) that is well suited for integration of synaptic responses. Somatodendritic KARs, acting through metabotropic signaling, regulate slow afterhyperpolarization current (I_{sAHP}) that directly impacts neuron excitability (Melyan et al., 2002, 2004).

KAR subunit expression can be detected in rat brain already during early embryonic development and persists throughout adulthood (Bahn et al., 1994). GluK1 subunit expression, in particular, is developmentally regulated and peaks during birth and early postnatal period (Bahn et al., 1994). Accordingly, GluK1-containing KARs exhibit developmentally regulated functions during the first postnatal week that coincides with the period of activity-dependent refinement of neuronal circuitry. By the third week after birth the immature-type GluK1-containing KARs are replaced by mature-type KARs (Maingret et al., 2005, Lauri et al., 2005, Vesikansa et al., 2012).

While neurodegenerative and neuropsychiatric disease pathogenesis involve a combination of a wide range of genetic, epigenetic and environmental factors, it is recognized that many of these illnesses exhibit abnormalities in the synaptic transmission. KARs, that effectively support neurotransmission, have been implicated in numerous central nervous system (CNS) disorders including anxiety, major depression, autism and epilepsy (reviewed by Contractor et al., 2011, Lerma and Marquez, 2013). However, current knowledge of KARs in human neurological disorders mainly originates from

genetic studies. Further studies are required to establish a detailed understanding of mechanisms connecting impaired KAR functions to possible disease onset and pathogenesis.

1.2.1 Somatodendritic KARs in hippocampus

Postsynaptic KARs were first described at the excitatory MF-CA3 synapse, where they localize to postsynaptic terminal and upon activation induce a low amplitude slowly decaying excitatory inward current ($EPSC_{KA}$) (Castillo et al., 1997; Vignes and Collingridge, 1997). Since then postsynaptic ionotropically active KARs have been found in CA1 interneurons where they mediate a relatively small $EPSC_{KA}$ and contribute to the interneuron excitability by depolarizing the cell membrane (Cossart et al., 1998; Frerking et al., 1998; Bureau et al., 1999; reviewed by Kullmann, 2001; Carta et al., 2014; Lerma and Marques, 2013; Evans and Henley, 2017; Akgul & McBain, 2016). Knock-out mice models combined with pharmacological studies have revealed that KARs in interneurons contain GluK1, GluK2, or both subunits (Cossart et al., 1998, Paternain et al., 2000, Mulle et al., 2000; Fisahn et al., 2004; Christensen et al., 2004; Wondolowski & Frerking, 2009). Recently CA1 interneuronal KARs have been reported to contain also the high-affinity subunit GluK5 (Wyeth et al., 2014). So far $EPSC_{KA}$ has not been described in CA1 pyramidal neurons.

Somatodendritic KARs regulate cellular excitability by activating an indirect signaling mechanism that regulates slow and medium afterhyperpolarizing currents (I_{sAHP} and I_{mAHP}, respectively). Such KARs have been found in both glutamatergic and GABAergic neurons in the adult hippocampus. In CA1 and CA3 pyramidal cells, GluK2-containing KARs regulate I_{sAHP} by activating a metabotropic

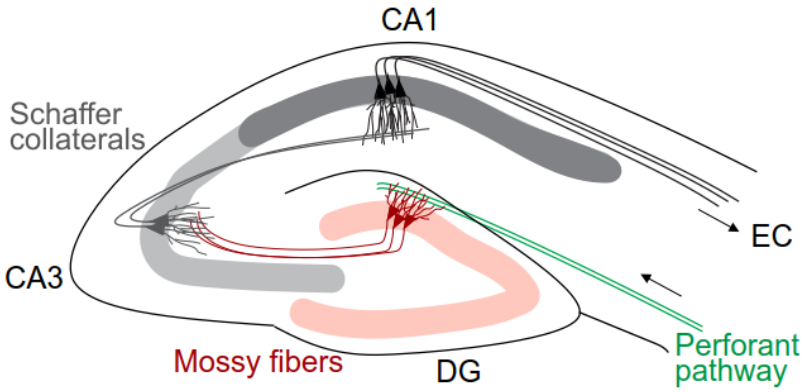


Figure 3. Schematic representation of glutamatergic circuitry in the hippocampus.

The scheme illustrates two main widely studied glutamatergic synapses connecting Dentate Gyrus (DG), Cornu Ammonis 3 (CA3; i.e. DG-CA3) and CA1 (i.e. CA3-CA1). Dentate Granule cells (red) located in DG (pink) receive inputs via Perforant pathway (green). The axons of Dentate Granule cells (i.e. Mossy Fibers) innervate glutamatergic pyramidal neurons (grey) in CA3. CA3 pyramidal neurons project their axons (Schaffer collaterals) to CA1, where they innervate CA1 pyramidal neurons (black). CA1 pyramidal neurons project to entorhinal cortex (EC). Hippocampus contains various types of interneurons that are not depicted here. (Adapted from Deng et al., 2010).

PKC-dependent signaling pathway (Melyan et al., 2002, 2004; Fisahn et al., 2005; Ruiz et al., 2005). In CA3 interneurons, somatodendritic GluK1-containing KARs regulate interneuron excitability by inhibiting I_{mAHP} in a G-protein dependent manner (Segerstrale et al., 2010). This property, however, is only present in the immature hippocampus during the first postnatal week.

1.2.2 Presynaptic KARs in hippocampus

KARs located at the presynaptic terminals typically regulate neurotransmitter release. Depending on the cell and synapse type, presynaptic KARs either inhibit or activate glutamate or GABA release (reviewed by Pinheiro and Mulle, 2008; Lerma and Marquez, 2013; Kullmann, 2001). In addition to regulating transmitter release, axonal KARs also affect axonal excitability (reviewed by Contractor, 2011).

Modulation of glutamate release by presynaptic KARs was first described at CA3-CA1 synapse, where KARs located in Schaffer collaterals depress glutamate release onto CA1 pyramidal neurons (Chitajallu et al., 1996; Kamiya and Ozawa, 1998; Vignes et al., 1997; Frerking et al., 2001; Clarke and Collingridge, 2002; Sallert et al., 2007). Using pharmacological tools it has been determined that presynaptic KARs at CA3-CA1 synapse contain GluK1 subunit (Vignes et al., 1998; Clarke and Collingridge, 2002). Since then presynaptic KARs have been described at Mossy Fiber synapses in area CA3 (Lauri et al., 2001; Schmitz et al., 2001) (Figure 3). At the Mossy Fiber synapse, KARs modulate glutamate release in a bidirectional manner that depends on the agonist concentration (reviewed by Lerma, 2003; Pinheiro and Mulle, 2006). In addition to regulating transmitter release, KARs present in Mossy Fibers affect also the axonal excitability of Dentate Granule cells (Kamiya and Ozawa, 2000).

KARs regulate GABA release from CA1 interneurons (reviewed by Kullmann, 2001; Lerma and Marques, 2013; Carta et al., 2014; Evans and Henley, 2017; Akgul & McBain, 2016). Similar to Mossy Fibers, presynaptic KARs regulating GABA release in CA1 have a bidirectional function where they can either inhibit or facilitate GABA release. Interestingly, the effect appears to depend on the cell type (Daw et al., 2010; Wyeth et al., 2017; Lourenco et al., 2010). KARs inhibit GABA release onto pyramidal cells (Clarke et al., 1997; Rodrigues-Moreno et al., 1997; Min et al., 1999; Maingret et al., 2005), while they facilitate or inhibit GABA release onto interneurons depending on agonist concentration (Cossart et al., 2001; Semyanov and Kullmann, 2001; Jiang et al., 2001). Whether the facilitatory action on GABA release involves KAR located extrasynaptically in axons or in the GABAergic terminals remains somewhat controversial (Cossart et al., 2001; Semyanov and Kullmann, 2001). In addition, inhibition of GABA release onto pyramidal cells includes a non-canonical signaling that is not implicated in facilitation of GABA release onto interneurons (Rodriquez-Moreno et al., 1998; Sihra and Rodriguez-Moreno, 2011).

1.3 KARs in the development of hippocampal neuronal circuits

KARs are expressed already in the embryonic development when initial synaptic contacts are formed in an activity independent manner. During this developmental period KARs are involved in regulating motility of axonal growth cone filopodia (Chang and De Camilli, 2001; Tashiro et al 2003), neurite outgrowth (Ibarretxe et al., 2007, Joseph et al., 2011, Marques et al., 2013) and mobilization of the

synaptic vesicles in the growth cones (Gelsomino et al., 2013). Presynaptically located KARs have been implicated in axon pathfinding and in the early phase of synaptic contact formation by affecting axonal filopodia and growth cone motility bidirectionally depending on the developmental stage (Tashiro et al., 2003). In an early stage KAR activation induces filopodia motility to promote contact finding. Whereas in a later stage, KAR signaling downregulates motility thereby promoting stabilization of the newly formed synaptic contacts and differentiation from filopodia to a mature synapse (Tashiro et al., 2003). In addition, somatodendritic GluK2-containing KAR activation promotes growth cone stalling and synaptic contact stabilization by transiently increased firing of action potential that propagate to the distant axonal growth cone (Ibarretxe et al., 2007).

In addition, KARs have been implicated in the development and maturation of glutamatergic synaptic connectivity (reviewed by Lauri and Taira, 2011, 2012) in particular in the area CA1 (Lauri et al., 2006; Vesikansa et al., 2007; Sakha et al., 2016) and in MF-CA3 synapse (Marchal and Mulle, 2004; Lanore et al., 2012). Pharmacological activation of native KARs increases functional glutamatergic connections to CA1 pyramidal neurons, while chronic antagonism of their endogenous activity results in reduced glutamatergic input (Vesikansa et al., 2007). Overexpression of recombinant KAR subunits has been shown to induce growth of axonal protrusions in cultured neurons (Sakha et al., 2016).

At the stage when formed synaptic contacts become functional, KARs are tonically activated by ambient glutamate (Lauri et al., 2005, 2006). These tonically active KARs (t-KARs) have been identified both in the principal cells (Lauri et al., 2005, 2006) and interneurons (Segerstråle et al., 2010). The immature type t-KARs modulate the activity dependent fine-tuning of the hippocampal circuitry and are therefore thought to be critical for the appropriate neuron circuit assembly (Vesikansa et al., 2007; Hanse et al., 2009; Lauri and Taira, 2011, 2012).

Tonically active KARs are activated by relatively low agonist concentrations (Lauri et al., 2006), raising the possibility that the receptor tetramer contains either or both of the high affinity subunits GluK4 and GluK5 (Figure 2). The physiological tonic activity can be studied by using pharmacological tools to block KARs. Using selective antagonists, it has been determined that t-KARs contain GluK1 subunit (Lauri et al., 2005, 2006). Messenger RNA expression studies have further suggested that GluK1 subunit splice variants could be differentially incorporated into t-KARs depending on the cell type. Pyramidal neurons express highly GluK1c, while GluK1b expression is restricted to interneurons (Vesikansa et al., 2012).

1.3.1 KARs at the immature CA3-CA1 synapse

In the CA3-CA1 synapse, t-KARs are present at the presynaptic terminal where they are continuously activated by ambient glutamate to maintain low probability of glutamate release (Lauri et al., 2006). Endogenous glutamate activates presynaptic t-KARs only during the first two weeks of development (Lauri et al., 2006). Tonically active KARs inhibit glutamate release by activating a non-canonical signaling pathway that does not involve the classical ionotropic action. Instead, t-KARs activate $G_{i/o}$-protein mediated cascade that targets potential regulators of neurotransmitter release (Lauri et al., 2006; Sallert et al., 2007).

Immature CA3-CA1 synapses with low release probability transmit with low efficacy and can undergo substantial frequency dependent facilitation of synaptic transmission (Lauri et al., 2006).

These type of synapses in particular are responsive to high frequency bursts of activity that is characteristic to developing neuronal networks (Lamsa et al., 2000; Palva et al., 2000). The loss of t-KARs are linked to functional maturation of CA3-CA1 synapses. The developmental shift from immature t-KAR to mature KAR activity involves BDNF/TrkB signaling (Sallert et al., 2009) and reduced affinity of the KARs (Lauri et al., 2006). Loss of presynaptic t-KARs is rapidly observed with induction of long-term potentiation (LTP) at the neonatal CA3-CA1 synapse (Lauri et al., 2006), while induction of long-term depression (LTD) in the area of CA1 leads to an upregulation of presynaptic t-KARs (Clarke et al., 2014).

Presynaptic KARs are present at CA3-CA1 synapse also later in development. However these mature-type receptors are no longer tonically active (Lauri et al., 2005; Maingret at al., 2005), likely due to a developmental switch in their subunit composition (Vesikansa et al., 2012).

1.3.2 KARs in immature CA3 interneurons

Although KAR expression is detected in CA3 interneurons throughout postnatal life (Bahn et al., 1994; Allen Mouse Brain Atlas, 2007), their physiological significance in immature CA3 interneurons remains elusive. Similar to adult hippocampus, KARs can be pharmacological activated at immature GABAergic interneurons, and have profound actions on interneuron excitability and GABAergic drive in the neonates (Maingret et al., 2005; Juuri et al., 2010; Lauri et al., 2005). The first studies describing the t-KAR dependent regulation of glutamate release in the hippocampus found no evidence for regulation of GABAergic transmission by endogenously active KARs (Lauri et al., 2005; Maingret et al., 2005). However, later Caiati and colleagues demonstrated that endogenous activation of presynaptic KARs reduces GABA release form Mossy Fiber terminals in the developing rat hippocampus (Caiati et al., 2010). In addition, Segerstrale and colleagues show t-KAR dependent regulation of CA3 interneuron firing rate in the immature hippocampus (Segerstrale et al., 2010). Similar to t-KARs in principal cells, tonically active KARs in CA3 interneurons have been proposed to contain GluK1 and GluK4/5 subunits (Segerstrale et al., 2010). Messenger RNA expression studies further suggest that GluK1 subunit splice variant GluK1b is selective to interneurons and could be incorporated to t-KARs present in immature CA3 interneurons (Vesikansa et al., 2012). Once activated by glutamate, t-KARs tonically inhibit I_{mAHP} by initiating a $G_{i/o}$-protein mediated signaling pathway that strongly modulates interneuron excitability ultimately leading to increased neuronal firing rate (Segerstrale et al., 2010). Tonically active KAR dependent I_{mAHP} modulation is downregulated during development leading to lower spontaneous action potential firing of mature CA3 interneurons (Segerstrale et al., 2010).

1.3.3 KARs and early network activity in the immature hippocampus

Activity-dependent fine-tuning is an essential developmental step for the emerging neuronal networks. Neuronal activity that underpins the synapse refinement process can be spontaneously generated by the network itself (Ben-Ari, 2001; Zhang and Poo, 2001; Spitzer, 2006; Kirkby et al., 2013). Spontaneous network bursts, characteristic for developing neuronal circuits, are also known as giant depolarizing potentials (GDPs) and can be observed both *in vitro* (Ben-Ari et al., 1989; Garaschuk et al., 1998; Lamsa et al., 2000) and *in vivo* (Leinekugel et al., 2002; Lahtinen et al., 2002).

Glutamatergic as well as GABAergic systems contribute to GDP generation, as they are both depolarizing during early development (Khazipov et al., 1997; Bolea et al., 1999; Lamsa et al., 2000; Ben-Ari et al., 2001). On one hand, GDPs can be blocked *in vitro* by using GABA receptor selective

antagonists, suggesting that GABAergic system is the primary driver for GDPs (Ganguly et al., 2001; Owens and Kriegstein, 2002; but see Lamsa et al., 2000). On the other hand, using $GABA_A$ blockers reveals a glutamatergic component of GDPs (Khazipov et al., 1997; Leinekugel et al., 1997; reviewed in Ben-Ari, 2007) and blocking fast glutamatergic transmission fully inhibits formation of GDPs (Bolea et al., 1999). GluK1-containing t-KARs have been shown to regulate spontaneous network bursts in the immature hippocampus (Lauri et al., 2005). In addition, a distinct population of high-affinity subunit containing KARs in CA3 pyramidal neurons induce neuronal firing and ultimately initiate network burst generation (Juuri et al., 2010). Interneurons play an instrumental role in synchronizing early hippocampal network bursts (Lamsa et al., 2000; Palva et al., 2000; Ben-Ari et al., 2004) raising a possibility that a subpopulation of t-KARs regulating CA3 interneuron firing could be responsible for the endogenous KAR dependent modulation of network bursts.

1.4 NETO proteins as auxiliary subunits of KARs

Neuropilin and tolloid-like (NETO) 1 and 2 have been identified as auxiliary subunits of native KARs (Zhang et al., 2009; Straub et al., 2011; Tang et al., 2011; Copits and Swanson, 2012). NETO1 was initially described to be an NMDA receptor (NMDAR) interaction partner (Ng et al., 2009), however this finding has not been confirmed by subsequent studies (Straub et al., 2011; Wyeth et al., 2014). NETO2 have been observed to bind also the potassium-chloride co-transporter KCC2 (Ivakine et al., 2013). By now it has been established that both NETO1 and NETO2 are critically involved in regulating various physiological properties of KARs (reviewed by Copits and Swanson, 2012).

NETO1 and NETO2 are single pass transmembrane proteins. N-terminal extracellular domain of NETOs contains two complement C1r/C1s, Uegf, Bmp1 (CUB) domains, which mediate interaction with other proteins such as KARs (Tang et al., 2011), and one low-density lipoprotein receptor class A (LDLa) domain (Figure 4) (Reviewed by Tomita and Castillo, 2012). Intracellular C-terminal domains of NETO proteins contain an AP2 adaptor protein endocytic motif. Other features of the C-terminus differ between NETO1 and NETO2. For example, NETO1 contains a type I PDZ ligand at the C-terminal end (Figure 4). Intracellular domain of NETO proteins contain potential phosphorylation sites that could additionally regulate NETOs and subcellular localization of the NETO/KAR complex (Lomash et al., 2017). NETOs have a brain specific complementary expression profile in the adult animals. NETO1 expression is restricted to hippocampus, being most pronounced in CA3 pyramidal neurons (Michishita et al., 2003, 2004; Ng et al., 2009; Straub et al., 2011). NETO2 expression, however, is predominant in most brain regions except the hippocampus (Straub et al., 2011).

Orthologues of vertebrate NETOs have been identified in *C. elegans* and *D. melanogaster* (Figure 4). NETO orthologues in *C. elegans* are three CUB-domain containing proteins SOL-1, SOL-2 and LEV-10 (Zheng et al., 2004, 2006; Wang et al., 2012). SOL-1 and SOL-2 are involved in modulation GLR-1 AMPA receptor function (Zheng et al., 2004, 2006, Wang et al., 2012), while LEV-1 modulates acetylcholine receptors (Gally et al., 2004). SOL-2 is most closely related and structurally similar to the vertebrate NETO2 protein (Figure 4) (Zhang et al., 2009; Wang et al., 2012). *Drosophila* Neto is obligatory for glutamate receptor clustering at the neuromuscular junction (NMJ) and essential for NMJ synaptic function (Kim et al., 2012, 2015; Ramos et al., 2015).

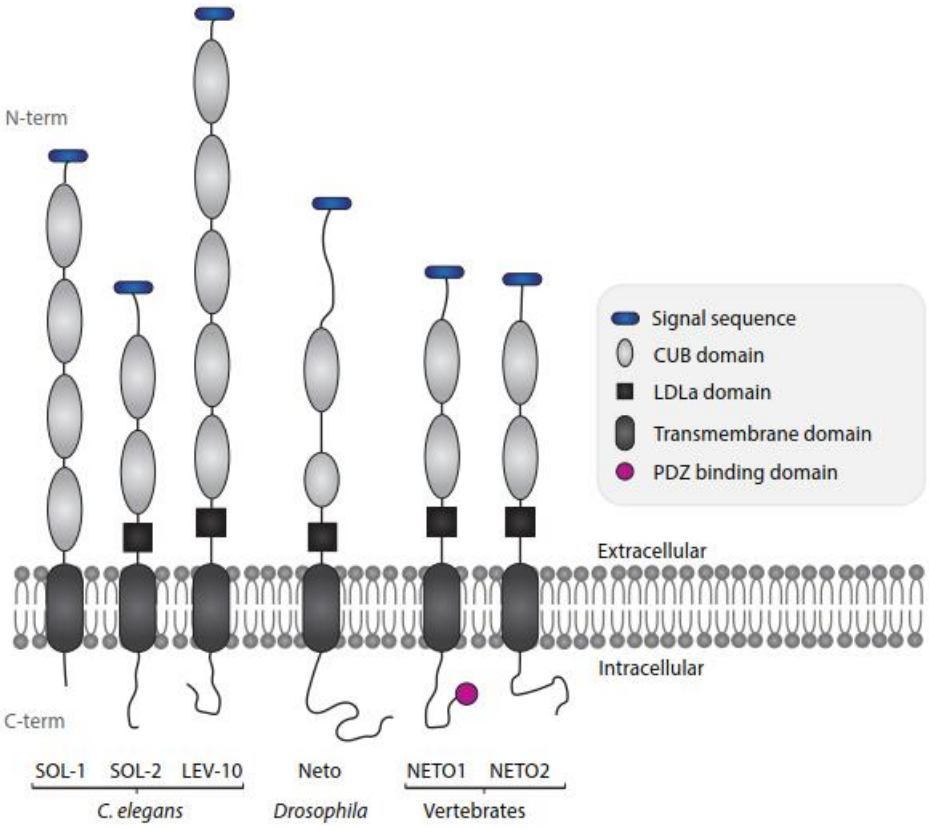


Figure 4. Structure of NETO proteins in different species.

NETOs are transmembrane proteins that consist of extracellular N-terminal domain, one transmembrane domain and intracellular C-terminal domain. Species specific differences determine the composition of N-terminal and C-terminal domains. The extracellular N-terminal domains containing two CUB domains and LDLa domain are similar in vertebrate NETO1 and NETO2. NETO1 C-terminus contains a PDZ binding domain (purple) that is not present in NETO2. In addition to mammals, NETO orthologues have been described in *C.elegans* (SOL-1, SOL-2, LEV-10) and *Drosophila* (Neto). (Modified from Copits and Swanson, 2012)

1.4.1 NETOs regulate subcellular localization of KAR subunits

NETOs are one of the key players implicated in regulation of KAR surface expression and subcellular localization (Tomita and Castillo, 2012). After their discovery NETO1 and NETO2 have been documented in numerous studies to regulate KAR subunit trafficking in both recombinant systems and in the native brain tissue.

Overexpression of NETO1 increases the surface expression and synaptic targeting of GluK1 subunit (Sheng et al., 2015; but see Copits et al., 2011). Loss of NETO1 was reported to cause a decrease in synaptic expression of native GluK2, GluK3 and GluK5 (Tang et al., 2011). However, another study did not see a significant change in the synaptic levels of GluK2 and GluK3 subunits in *Neto1* knock-out mice (Straub et al., 2011). Nevertheless, NETO1-dependent reduction of postsynaptic native GluK2/3 subunits were later confirmed in a detailed electron microscopic study at MF-CA3 synapse (Wyeth et al., 2014).

NETO2 enhances the surface expression of GluK1, but does not increase the surface levels of GluK2 in heterologous systems (Zhang et al., 2009). NETO2 also promotes GluK1 surface expression and synaptic targeting in hippocampal neurons (Copits et al., 2011; Sheng et al., 2015), depending on phosphorylation of NETO2 at serine 409 (Lomash et al., 2017). Additionally, it has been proposed in a recent study that GluK2 subunit has a strong forward trafficking N-terminal signal that is independent of NETO interaction, while the GluK1 subunit contains a forward trafficking repressor motif that is likely masked by NETO1 binding (Sheng et al., 2017).

Taken together, the existing evidence suggests that NETO1 is non-selectively promoting trafficking of different KAR subunits, while NETO2 appears to selectively regulate subcellular localization of GluK1 subunit. Whereas, synapse and cell type difference of NETO/KAR trafficking is yet to be resolved.

1.4.2 NETOs regulate biophysical properties of KARs

Native KARs exhibit a characteristically slow current kinetics that set them apart from recombinant KARs and native AMPA receptors (AMPAR) (Vignes and Collingridge, 1997; Castillo et al., 1997). Indeed, most recombinant KARs in heterologous systems display fast activation, deactivation and desensitization kinetics (Heckmann et al., 1996; Swanson et al., 2002; Bowie et al., 2003). However, a recombinant KAR heteromer containing GluK2/5 have been found to display slow current kinetics comparable to native synaptic KARs (Barberis et al., 2008).

NETO1 and NETO2 involvement in regulation of the biophysical properties of KAR subunits have been addressed by overexpressing NETO and KAR subunits in cell-lines and cultured hippocampal neurons. NETO1 affects KAR current kinetics in a subunit dependent manner. NETO1 interaction speeds GluK1 desensitization (Copits et al., 2011; Sheng et al., 2015) and reduces the onset of desensitization and speeds recovery from desensitization of GluK2-containing, GluK2/4- and GluK2/5-containing KARs (Fisher and Mott, 2013). The presence of NETO2 increases the open probability of recombinant GluK2 (Zhang et al., 2009), speeds GluK2 recovery from desensitization (Fisher et al., 2015) and determines the slow deactivation of homomeric GluK2 and heteromeric GluK2/5 (Griffith and Swanson, 2015). NETO2 interaction with GluK1 slows the receptor deactivation and desensitization (Sheng et al., 2015; Fisher et al., 2015), thus having an opposite effect on GluK1 kinetics as compared to NETO1. The differential regulation of recombinant GluK1 desensitization is determined by the N-terminal CUB domains of NETO1 and NETO2 (Fisher et al., 2015).

A few studies addressing the NETO-dependent regulation of native KAR biophysical properties support findings obtained from recombinant systems. The auxiliary subunit NETO1 determines the slow kinetics of synaptic KARs (Straub et al., 2011; Tang et al., 2011). Loss of NETO1 accelerates the rise time and decay kinetics of KAR-mediated synaptic current at MF-CA3 synapses (Straub et al., 2011; Tang et al., 2011).

1.4.3 NETOs regulate agonist affinity of KARs

The distinctive distribution of native KARs in the hippocampus was originally demonstrated with [^{3}H] kainate labelling (Foster et al., 1981; Monaghan & Cotman, 1982) and later confirmed with GluK2 knock-out mice (Mulle et al., 1998). Interestingly, a strong reduction of [^{3}H] kainate binding was observed in the hippocampus of *Neto1* knock-out mice (Straub et al., 2011). Indeed, NETO1 increases KAR affinity to kainic acid both in the brain and in heterologous cells expressing GluK2 and GluK5 (Straub et al., 2011). Similarly, NETO2 increases kainic acid affinity of both recombinant GluK2 and native GluK2 subunits (Zhang et al., 2009). Both NETO1 and NETO2 interaction with recombinant GluK1 cause a substantial increase in glutamate sensitivity, while NETO2 has a modest effect on glutamate affinity of GluK2 homomeric receptor (Fisher et al., 2015).

So far, the effect of NETOs on agonist affinity of KARs has been assessed with regards to kainic acid and glutamate (Straub et al., 2011; Fisher and Mott, 2013; Wyeth et al., 2017). Further studies are needed to test whether NETO interaction affects sensitivity and selectivity of other KAR agonists and antagonists.

1.4.4 Physiological significance of NETO/KAR interaction in adult animals

In parallel to studies using recombinant receptors, transgenic mouse models where *Neto1*, *Neto2*, or both have been knocked out were generated (Ng et al., 2009; Tang et al., 2011). NETO deficient mouse models have been instrumental in elucidating the physiological significance of NETOs on synaptic transmission, neuronal network activity and the animal behavior (Ng et al., 2009; Straub et al., 2011; Tang et al., 2011; Wyeth et al., 2017; Mennesson et al., 2019).

Neto1KO mice exhibit compromised spatial memory, consistent with altered hippocampal function (Ng et al., 2009). Additionally, NETO1 deficiency is associated with impaired kainate-induced gamma oscillations in the hippocampus *in vitro* (Wyeth et al., 2017). Compromised kainate-induced gamma oscillations in CA3 hippocampus are proposed to reflect NETO1-dependent reduction of functional KARs in GABAergic neurons (Wyeth et al., 2017), while the impaired spatial learning may also relate to loss of KAR functions at MF-CA3 synapse (Straub et al., 2011; Tang et al., 2011). Neto2KO mice have reduced fear expression and extinction in cued fear conditioning that points towards physiologically important NETO2 role in fear-related brain regions including amygdala, hippocampus and medial prefrontal cortex (Mennesson et al., 2019). This phenotype was associated with reduced synaptosomal KARs from the said brain regions (Mennesson et al., 2019). Accordingly, mice lacking different KAR subunits display aberrant anxiety-like behaviors (Wu et al., 2007; Shaltiel et al., 2008; Catches et al., 2012).

KAR subunits have been associated with developmentally originating neuropsychiatric disorders including obsessive-compulsive disorder (Mattheisen et al., 2015), bipolar disorder, major depression, and schizophrenia (Beneyto et al., 2007). Defining the roles of NETO auxiliary subunits might resolve critical poorly understood aspects related to KAR functions in normal and pathological circuit development and function.

2. AIMS

The main goal of this thesis was to elucidate the molecular mechanisms by which NETOs modulate KARs during the development of synaptic circuitry in the hippocampus. Specifically, the aim was to

1. Study the role of NETO1 and NETO2 in the subcellular localization of KAR subunits in cultured glutamatergic and GABAergic neurons (I, II)

2. Clarify the role of NETO/KAR complex in formation and maturation of glutamatergic synapses in cultured glutamatergic and GABAergic neurons (I, II)

3. Elucidate the role of NETO1 in regulating presynaptic KAR functions at immature CA3-CA1 synapse (I)

4. Characterize the role of NETO1 in regulating postsynaptic KARs in CA3 *stratum radiatum* interneurons during circuit maturation (II)

3. MATERIALS AND METHODS

This chapter gives an overview of the materials and methods the author personally employed in this thesis (Table 1). The author was not involved in carrying out the following experiments: quantitative PCR (I), *in situ* hybridization (I), preparing organotypic slice cultures (I), microelectrode array recordings (I), and spontaneous network activity recordings (II). A detailed description of all experimental procedures is covered in the original publications. All animal experiments were approved by the Ethics Committee and performed in accordance with the University of Helsinki Animal Welfare Guidelines.

Table 1. List of methods used. Only those methods where the author was personally involved are listed here.

METHOD	PUBLICATION
Acute slice preparation	I, II
Primary hippocampal neuron culture	I, II
Electrophysiology	I, II
gDNA extraction and genotyping	I, II
cDNA synthesis and RT-PCR	I
Plasmid generation	I
Production of lentivirus particles	I
Lentiviral infection of cultured neurons	I, II
Immunofluorescence	I, II
Confocal imaging and image analysis	I, II
Statistical analysis	I, II

3.1 Preparations used in this study

3.1.1 Transgenic mouse lines

Male and female Wild-type (WT), *Neto1* knock-out (Neto1KO) and *Neto2* knock-out (Neto2KO) (C57Bl/6NCrl) mice (Ng et al., 2009; Tang et al., 2011) were used in this study. The transgenic mouse lines were kindly provided by Roderick McInnes (McGill University, Montreal, Canada).

3.1.2 Acute hippocampal slices

Acute parasagittal hippocampal sections were prepared from brains of postnatal day (P) 4-P6 and P14-P16 WT, Neto1KO and Neto2KO mice using standard methods (Lauri et al., 2006). In publication I, 400 µm thick acute slices were prepared using a dissection solution containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $CaCl_2$, 26 $NaHCO_3$, 10 $MgSO_4$, 15 D-glucose. In publication II, 350 µm thick acute slices were prepared using sucrose dissection solution containing (in mM): 87 NaCl, 2.5 KCl, 7 $MgCl_2$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 25 $NaHCO_3$, 75 D-sucrose, 25 D-glucose and equilibrated with 95% O_2 and 5% CO_2. High sucrose in dissection solution helps to maintain the viability of surface neurons and is preferred in visually guided whole-cell patch clamp experiments. Immediately after cutting, the slices were transferred to artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, 26 $NaHCO_3$, 15 D-glucose and incubated 30 min at +35ºC and 30 min - 4h at room temperature before use.

3.1.3 Cultured primary hippocampal neurons

Primary hippocampal neurons were obtained from P0-P2 WT, Neto1KO and Neto2KO mouse pups. Hippocampi were isolated from the brain, digested with papain (500 µg/ml, Sigma) and triturated. Cells were counted and plated with a density of 10000 cells/µm on poly-L-lysin (Sigma) coated 24-

well plates containing glass coverslips. Hippocampal neurons were grown in Neurobasal A (Gibco) medium containing 2% B27 supplement, 0.5 mM L-glutamine and 1% penicillin/streptomycin (all from Life Technologies). Some of the cultures were infected with lentiviral vectors at 3 days-in-vitro (DIV3) and fixed at DIV14 using 4% PFA in PBS. Fixed cultures were kept at +4ºC until further analysis. See Figure 5 A for schematic representation of cell culture experiments.

3.2 Electrophysiology

3.2.1 Whole-cell voltage clamp

Whole-cell patch clamp recordings were done from CA1 pyramidal neurons (I) or CA3 *stratum radiatum* interneurons (II) in acute or cultured hippocampal slices. Differential interference contrast (DIC) optics were used to visually identify CA3 *stratum radiatum* interneurons. During the recordings, the chamber was continuously perfused with ACSF (32ºC) bubbled with 95% O2 and 5% CO2.

Glutamatergic currents were recorded with 3-5 MΩ glass electrodes filled with Cs-based intracellular solution containing (in mM): 130 CsMeSO4, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 5 QX-314, 8 NaCl; 285 mOsm (pH 7.2). To record medium afterhyperpolarizing current (I_{mAHP}), the filling solution contained (in mM): 130 K-gluconate, 10 HEPES, 10 KCl, 4 ATP-Mg, 0.3 GTP-Mg, 0.2 EGTA; 285 mOsm (pH 7.2). I_{mAHP} was induced by applying depolarizing 60 mV 40 ms step from a holding potential -47 mV.

EPSCs were evoked by afferent stimulation with a basal frequency of 0.05 Hz. AMPAR-KAR mediated responses were recorded in the presence of 100 µM picrotoxin (Abcam) and 50 µM D-AP5 (HelloBio) to antagonize $GABA_A$- and NMDA receptors, respectively, at holding potential -70 mV. NMDAR-mediated responses were recorded at a holding potential +40 mV and in the presence of AMPA/KAR antagonist CNQX (10 µM, Abcam) and 100 µM picrotoxin. 1 µM tetrodotoxin (TTX, Abcam) was added to the drug cocktail for recording of spontaneous action potential independent miniature excitatory postsynaptic currents (mEPSC). In order to isolate KAR component of the evoked response, AMPAR selective antagonist GYKI53655 (30 µM) was added to the bath solution. In some experiments GluK1 specific antagonist ACET (200 nM, Tocris) and agonist ATPA (1 µM, Tocris) were used. Table 2 covers the details of the used pharmacological tools.

In all experiments, uncompensated series resistance (Rs < 30 MOhm) was monitored, and cells were discarded if Rs varied more that 20%. Data were collected using Axoscope 9.2 (Axon instruments) or WinLTP software (Anderson and Collingridge, 2007). Spontaneous events were analyzed with MiniAnalysis 6.0.3 program (Synaptosoft Inc.). Events were verified visually, and events with amplitude less than three times the baseline rms noise level were rejected. Evoked EPSC and I_{mAHP} amplitude was analyzed using WinLTP and calculated in 1 min bins. Holding current data were collected during I_{mAHP} recordings and analyzed using WinLTP.

3.2.2 Cell attached recording

Spontaneous action potential firing was measured from CA3 *stratum radiatum* interneurons in cell attached configuration using ACSF filled 10 MΩ microelectrodes.

Table 2. Pharmacological tools used in this study.

DRUG	CONCENTRATION	ACTION	PUBLICATION
Picrotoxin	100 µM	$GABA_A$ receptor antagonist	I, II
D-AP5	50 µM	NMDA receptor antagonist	I, II
TTX	1 µM	Na^+-channel blocker, blocks action potentials	I, II
CNQX	10 µM	AMPA/KAR antagonist	II
GYKI53655	30 µM	AMPA receptor antagonist	II
ACET	200 nM	GluK1 selective KAR antagonist	I, II
ATPA	1 µM	GluK1 selective KAR agonist	I, II

3.3 Molecular biology

3.3.1 cDNA synthesis and RT-PCR

Cultured hippocampal neurons were collected and subjected immediately for total RNA purification. RNA purification was done using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using oligo(dT)18 primer and RevertAid First Strand cDNA synthesis Kit (Thermo Scientific).

Neto1 and *Neto2* expression was detected using Phusion High-Fidelity DNA polymerase (#F-530L, Thermo Scientific) according to manufacturer's recommendation. *Gadph* was used as a reference gene. Reverse transcriptase PCR (RT-PCR) primers (Table 3) were designed by the author and ordered from Oligomer oy. PCR products were visually confirmed by gel electrophoresis.

Table 3. List of primers used in this study

TARGET	FORWARD	REVERSE	APPLICATION
Neto1	TGAGTTTGAGATGGGCGGCC	ACTGGTGTTGGTCAGCTGAT	RT-PCR
Neto2	CTGATGGAATAGTGCGGTCT	GATCGTCCCATGAGTCTTCG	RT-PCR
Gapdh	CAACGACCCCTTCATTGACC	AGTGATGGCATGGACTGTGG	RT-PCR
mRtl15UTR-F	AGATCGGAGCCTCTGGTGTAAC	-	Genotyping
mRtl1intron-R	-	GGATTACGTGAATCTCTTAACTG	Genotyping
pcDNA3tau-R	-	TTACTGACCATGCGAGCTTG	Genotyping
mRtl2-2larm-F2	GTAGGTATAGGTAGGATGGTT	-	Genotyping
mRtl2-intron-R	-	GCAGAAGTACCAGAAAGC	Genotyping
DTA-R2	-	CTAGTGAGACGTGCTACTTC	Genotyping
M13	GTAAAACGACGGCCAGT	CAGGAAACAGCTATGAC	Colony PCR
rat *Neto1*	ATGATCTATGGACGCAGTTTG	TTAGACCCTAGTTGTGTTGTA	Cloning
rat *Neto2*	ATGGCCCTGGAGCAGCTCT	TTAAAAGTCGATGGATATGGAC	Cloning
Neto1 3' Xho	GCCACCATGATCTATGGACGCAGTTTG	*CTCGAG*ATAGACCCTAGTTGTGTTGTA	Cloning
Neto2 3' Xho	GCCACCATGGCCCTGGAGCAGCTCT	*CTCGAG*ATAAAAGTCGATGGATATGGA	Cloning
HA-tag 5' Xho	*CTCGAG*ACTGGAGGATAC	GGCCGTTACTAGTGGATC	Cloning

3.3.2 gDNA extraction and genotyping

Genomic DNA (gDNA) was extracted from the ear samples of transgenic *Neto1* or *Neto2* mice. RT-PCR was performed using Multiplex PCR kit (Qiagen) according to the manufacturer's instructions. *Neto1* samples were amplified using mRtl15UTR-F, mRtl1intron-R, and pcDNA3tau-R (Table 3).

Neto2 samples were amplified using mRtl2-2larm-F2, mRtl2-intron-R, and DTA-R2 (Table 3). The PCR products were analyzed by gel electrophoresis.

3.3.3 Plasmid generation and lentivirus production

Lentiviral constructs used in this study are listed in Table 4. Epitope tagged plasmid constructs for NETO1 and NETO2 were generated by the author. *Neto1* and *Neto2* were amplified by PCR from rat brain cDNA using primers listed in Table 3. *Neto1* and *Neto2* cloning forward primers contained a Kozak sequence (Table 3; underlined) in front of a start codon. *Neto1* and *Neto2* reverse primers contained an in-frame XhoI site (Table 3; shown in italics) for subsequent C-terminal HA-tag addition. Sequence coding HA-tag was amplified from Addgene plasmid #10792 (a gift of William Sellers) using a forward primer that contained in-frame XhoI site. The PCR products were inserted into pTZ57R/T cloning vector (Thermo Scientific) using TA-cloning. In-frame HA-tag was added to the 3'-end of *Neto1* and *Neto2* using a restriction enzyme treatment followed by ligation. The epitope tagged constructs were sub-cloned into a second generation lentiviral vector (Zufferey et al., 1997). Plasmids encoding various KAR subunits were described previously (Vesikansa et al., 2012; Sakha et al., 2016). The lentiviral particles were prepared as described (Vesikansa et al., 2012). Briefly, lentiviral particles were produced by transfecting HEK293FT cells with lentiviral construct and helper plasmids using JetPEI (Polyplus transfection). Cell culture supernatant was collected and virus particles precipitated using PEG-it (System Biosciences). Precipitated virus particles were aliquoted and stored at -80ºC until further use. Neuron cultures were infected by adding virus particles to culture media.

Table 4. List of lentiviral constructs used in this study (Modified from Publication I).

PROTEIN	PROMOTER	TAG	TAG LOCATION	PUBLICATION
GluK1b	CMV	flag	N-terminus, after signal sequence	I, II
GluK1c	CMV	flag	N-terminus, after signal sequence	I, II
GluK2	CMV	myc	N-terminus, after signal sequence	I, II
GluK4	CMV	myc	N-terminus, after signal sequence	I
GluK5	CMV	myc	N-terminus, after signal sequence	I
NETO1	CMV	HA	C-terminus	I
NETO2	CMV	HA	C-terminus	I

3.4 Immunofluorescence

Fixed neurons were permeabilized using 0.2% Triton X-100 in PBS, followed by 1 h blocking at room temperature. Blocking solution contained typically 5% goat serum, 2% bovine serum albumin (BSA), 0.1% Triton X-100 and 0.05% Tween-20 in PBS. Primary antibodies (Table 5) were diluted in the blocking solution (II) or a carrier solution (I) containing 1% BSA and 0.1% gelatin in PBS. Samples were incubated with primary antibodies over-night at +4ºC. Secondary antibodies (Table 6) were diluted in PBS and incubated 1-1.5 h at room temperature. The processed samples were mounted on glass microscope slides using Prolong Gold antifade reagent (P36934, Life Technologies).

3.5 Confocal imaging and image analysis

All samples were blinded for genotype and KAR/NETO subunit expression during staining, image acquisition and analysis. Confocal images of neurites and neuron morphology were acquired using a LSM Zeiss 710 confocal microscope (alpha Plan-Apochromat 63x/1.46 OilKorr M27 objective).

High-resolution images of synaptic structures were collected using Leica TCS SP8 confocal microscope and HC PL APO 93x/1.30 motCORR STED WHITE (glycerol) and 3x digital zoom.

Synaptophysin puncta density, axonal and dendritic delivery of overexpressed KARs or NETOs were analyzed using MATLAB/SynD (Schmitz et al., 2011) (I, II). NETO-HA colocalization with Synaptophysin was analyzed using ImageJ software (I). Synaptic recruitment of KARs, synapse cluster density, AMPAR/KAR containing synapses were analyzed from the high-resolution images using Imaris software (II).

Table 5. List of primary antibodies used in this study (Modified from Publication II).

PRIMARY ANTIBODIES				
Antibody	**Dilution**	**Product nr**	**Manufacturer**	**Publication**
Guinea pig anti-Synaptophysin	1:2000	101004	Synaptic Systems	I, II
Mouse anti-PSD95	1:1000	75-028	NeuroMab	II
Chicken anti-GAD67	1:2000	198006	Synaptic Systems	II
Mouse anti-GAD67	1:1000	MAB5406	Millipore	II
Rabbit anti-GAD67	1:2000	198013	Synaptic Systems	II
Mouse anti-flag	1:1000	F1804	Sigma Aldrich	I
Rabbit anti-flag	1:1000	F7425	Sigma Aldrich	II
Rabbit anti-myc	1:1000	06-549	Millipore	I, II
Mouse anti-HA	1:1000	MMS-101R	Covance	I
Chicken anti-MAP2	1:8000	AB5543	Millipore	I, II
Mouse anti-GluA2/4	1:2500	MAB396	Millipore	II

Table 6. List of secondary antibodies used in this study (Modified from Publication II).

SECONDARY ANTIBODIES				
Antibody	**Dilution**	**Product nr**	**Manufacturer**	**Publication**
Goat anti-chicken Alexa Fluor 405	1:2000	ab175674	Abcam	I, II
Goat anti-mouse Alexa Fluor 488	1:2000	A11029	Life Technologies	II
Goat anti-guinea pig Alexa Fluor 568	1:2000	A11075	Life Technologies	I, II
Goat anti-rabbit Alexa Fluor 647	1:2000	A-21245	Molecular Probes	I, II
Goat anti-mouse Alexa Fluor 647	1:2000	A21236	Life Technologies	I, II

3.6 Statistical analysis

All statistical analysis was performed on raw data using Sigma-Plot software. First, the normal distribution of the data was tested with Shapiro-Wilk test. Then, one-way ANOVA with Holm-Sidak post hoc comparison or Kruskal-Wallis test was used accordingly. Student's paired t-test was used to assess the treatment effect within a group. All data are presented as mean ± SEM; $p < 0.05$ was considered statistically significant. In figures, the significance levels are indicated by asterisks as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4. RESULTS AND DISCUSSION

4.1 The role of NETOs in the subcellular localization of KARs (I, II, unpublished data)

4.1.1 Expression and subcellular localization of NETO1 and NETO2

Expression of NETOs has been mainly studied in the adult brain (reviewed by Copits and Swanson, 2012). Michishita and colleagues demonstrated that NETO expression in different brain regions is developmentally regulated (Michishita et al., 2003, 2004), however these studies lacked spatial resolution. Therefore, we confirmed *Neto1* and *Neto2* expression in hippocampus during early postnatal development using quantitative PCR (qPCR), reverse transcriptase PCR (RT-PCR) and *in situ* hybridization. Both *Neto1* and *Neto2* were expressed in hippocampus at postnatal day 4 (P4) but showed distinct region specific expression patterns. *Neto1* expression was mainly restricted to CA3, while *Neto2* showed broader expression in principal neurons within all the hippocampal subfields. Our *in situ* hybridization results further indicated that both *Neto*s can be expressed in putative interneurons located in *stratum radiatum*. Importantly, we found that under our culture conditions, *Neto1* and *Neto2* expression can be detected also from WT primary hippocampal neurons throughout the two-week long culture period.

In order to study the subcellular distribution of NETO1 and NETO2, without available specific antibodies against native NETO1 and NETO2, we generated HA-tagged NETO1 and NETO2 and overexpressed them in cultured primary hippocampal neurons. Both HA-tagged NETOs distributed to axons and dendrites of cultured neurons. In addition, both overexpressed NETOs co-localized with the presynaptic marker Synaptophysin (Syn).

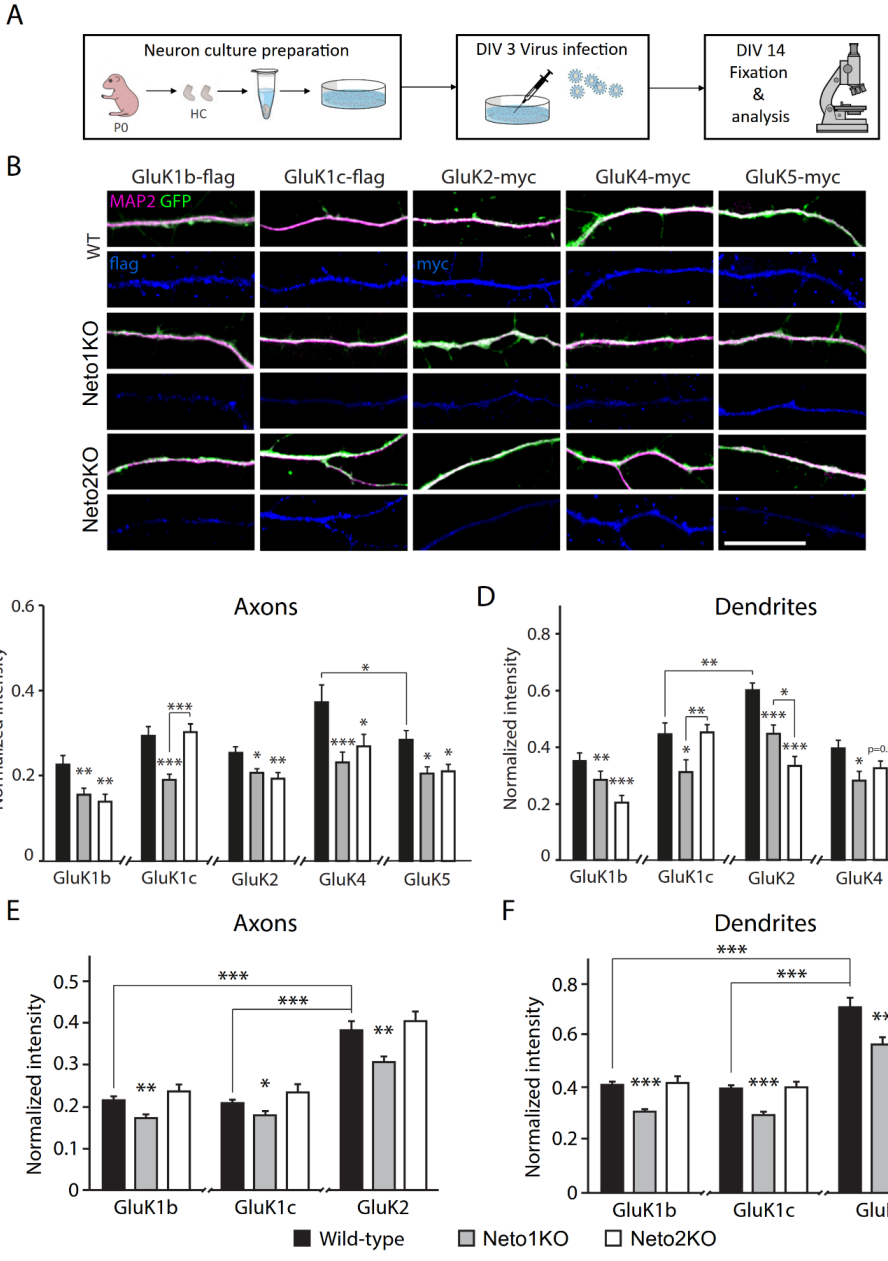
Taken together, these findings indicate that *Neto1* and *Neto2* are expressed in hippocampus already at early postnatal development. Overexpressed recombinant NETO1 and NETO2 can localize to both dendrites and axons, including putative presynaptic release sites. Thus, these data provide a proof-of-concept for the following experiments exploring NETO proteins in the subcellular localization of KAR subunits.

4.1.2 Axonal and dendritic targeting of KARs in NETO-deficient pyramidal neurons

KARs can localize to both axons and dendrites of hippocampal pyramidal neurons (reviewed by Contractor et al., 2011). NETOs are known to regulate subcellular localization of KAR subunits (Copits and Swanson, 2011), however synapse and cell type dependent nuances remain unclear. In particular, no previous data on the role of NETOs in axonal targeting of KARs exists.

Selective antibodies to reliably distinguish between different native KAR subunits in cultured neurons are not available. Therefore, to study the effect of NETO1 and NETO2 on axonal and dendritic targeting of KARs, we overexpressed myc- and flag-tagged recombinant KAR subunits in cultured hippocampal neurons from WT, Neto1KO and Neto2KO mice (Figure 5 A). KAR subunits GluK1, 2, 4 and 5, including GluK1 splice variants GluK1b and GluK1c were included in the analysis.

In WT glutamatergic neurons, all studied KAR subunits were detected in distal MAP2 negative axons (>150 µm from the soma), with flag-GluK1c and myc-GluK4 showing the highest relative intensity in this region (see also Vesikansa et al., 2012). Distal MAP2 positive dendrites also contained all studied KAR subunits (Figure 5 B), with myc-GluK2 having the highest relative intensity (Orav and Lauri, unpublished data).


A
Neuron culture preparation
P0
HC
DIV 3 Virus infection
DIV 14
Fixation
&
analysis
B
GluK1b-flag
GluK1c-flag
GluK2-myc
GluK4-myc
GluK5-myc
MAP2 GFP
flag
myc
WT
Neto1KO
Neto2KO
C
Axons
Normalized intensity
GluK1b
GluK1c
GluK2
GluK4
GluK5
D
Dendrites
p=0.07
p=0.09
E
Axons
F
Dendrites
Wild-type
Neto1KO
Neto2KO

◀**Figure 5.** Targeting of KAR subunits in cultured hippocampal neurons.

A. Outline of neuron culture experimental procedure.
B. Example images of KAR subunit (blue) targeting to MAP2 (purple) positive dendrites in GFP expressing (green) WT, Neto1KO and Neto2KO neurons. MAP2 and GFP signal is shown as merged. Scale bar 20 μm.
C. Pooled data of KAR subunit targeting to axons of Neto1KO and Neto2KO neurons, shown as % of WT. Flag-GluK1b n=47 and n=24, flag-GluK1c n=13 and n=29, myc-GluK2 n=41 and n=34, myc-GluK4 n=18 and n=22, myc-GluK5 n=34 and n=48 for Neto1KO and Neto2KO, respectively.
D. Pooled data of KAR subunit targeting to dendrites of corresponding Neto1KO and Neto2KO neurons shown in C.
E. Pooled data of KAR subunit targeting to axons of GAD67+ WT, Neto1KO and Neto2KO neurons. For quantification, the GluK signal intensity in the neurites is normalized to the soma intensity. GluK1b-flag (n = 49, n = 34, n = 12), GluK1c-flag (n = 49, n = 33, n = 12), and GluK2-myc (n = 43, n = 32, n = 18) in WT (black bar), Neto1KO (grey bar) and Neto2KO (white bar) neurons, respectively.
F. Pooled data of KAR subunit targeting to dendrites of corresponding GAD67+ WT, Neto1KO and Neto2KO neurons. GluK1b (n = 49, n = 34, n = 18), GluK1c (n = 49, n = 33, n = 15), and GluK2 (n = 42, n = 32, n = 18) in WT (black bar), Neto1KO (grey bar), and Neto2KO (white bar) neurons, respectively.

NETO1 and NETO2 deficiency impaired axonal and dendritic targeting of most KAR subunits to a similar extent, suggesting a mostly non-selective role, with a few exceptions. Axonal and dendritic delivery of GluK1c was selectively reduced in Neto1KO, but not in Neto2KO, neurons (Figure 5 B, C, D) (I).

The axonal and dendritic delivery of GluK2 is significantly lower in both Neto1KO and Neto2KO neurons in comparison to WT (Figure 5 B, C, D) (I). Interestingly, as compared to NETO1, loss of NETO2 has a significantly larger effect on dendritic delivery of GluK2. The axonal delivery of GluK4 is significantly impaired in both Neto1KO and Neto2KO principal neurons when compared to WT. Whereas GluK4 dendritic targeting appears to involve NETO1, but not NETO2 (Figure 5 B, C, D) (Orav and Lauri, unpublished). Finally, dendritic targeting of GluK5 was not affected by the absence of NETO1, while loss of NETO2 significantly impaired GluK5 delivery to distal dendrites (Figure 5 B, C, D) (Orav and Lauri, unpublished). Taken together these data suggest a selective NETO1-dependent regulation of axonal GluK1c, dendritic GluK1c and dendritic GluK4. In addition, the findings indicate a selective NETO2-dependent regulation of dendritic GluK2 and GluK5 subunit.

4.1.3 Targeting and synaptic recruitment of KARs in NETO-deficient GAD67+ neurons

Although both NETOs are expressed in interneurons in the neonatal (Orav at al., 2017) and adult hippocampus (Straub et al., 2011; Wyeth et al., 2017), the role of NETOs in targeting of various KAR subunits has so far been studied only in glutamatergic neurons (Copits et al., 2011; Tang et al., 2011; Straub et al., 2011; Wyeth et al., 2014; Sheng et al., 2015, 2017; Orav et al., 2017).

To address the role of NETOs in regulating KAR subunit targeting in GABAergic neurons we overexpressed tagged GluK subunits in cultured hippocampal WT, Neto1KO and Neto2KO neurons, where 9% of the cells are GABAergic as identified with GAD67+ labeling. Recombinant KAR subunits GluK1b, GluK1c and GluK2 were targeted to MAP2 negative axons and MAP2 positive dendrites in WT GAD67+ neurons (Figure 5 E, F). Both axonal and dendritic delivery of the three

subunits GluK1b, GluK1c, GluK2 were significantly lower in the dendrites of cultured GAD67+ Neto1KO neurons as compared to controls (Figure 5 E, F). Interestingly, the targeting of the studied KAR subunits was not changed in cultured GAD67+ Neto2KO neurons when compared to WT controls (Figure 5 E, F) suggesting that KAR subunit delivery in GABAergic neurons is selectively dependent on NETO1.

NETO1 has been implicated in promoting the synaptic targeting of KARs in a subunit dependent manner in glutamatergic principal neurons (reviewed by Tomita and Castillo, 2012). However, the subcellular compartmentalization of NETO1/KAR complex in GABAergic interneurons has not been previously characterized. To further dissect the NETO1-dependent subcellular localization of KAR subunits in GABAergic neurons, we analyzed the distribution of GluK subunits between synaptic and extrasynaptic pools in WT and Neto1KO GAD67+ neurons. KAR puncta were considered synaptic when they co-localized with synapse clusters identified by Synaptophysin (Syn) and PSD95 co-labelling (see Methods section in publication II for details). Only a minority of the recombinant overexpressed KAR subunits co-localized with Syn-PSD95 clusters. Loss of NETO1 did not affect the synaptic recruitment of interneuron specific GluK1b and GluK2 in GAD67+ neurons. However, the amount of synaptic GluK1c was significantly lower in Neto1KO interneurons as compared to controls.

4.1.4 Subunit and cell type dependent effects of NETO on KAR targeting

Earlier studies have provided insight to the possible mechanisms underlying NETO regulation of KAR subunit targeting (reviewed by Copits and Swanson, 2011). However, details regarding subcellular compartment, subunit and cell type specific mechanisms have been unresolved. Our data show that the NETO regulation of KAR targeting depends not only on the KAR subunit identity, but also on NETO isoform and neuron type.

In the principal neurons, both NETO1 and NETO2 contributed to targeting of most KAR subunits, except for GluK1c which was selectively regulated by NETO1. In contrast, in GABAergic neurons, the trafficking of KAR subunits was not affected by NETO2 (Figure 5 E, F). These findings suggest a NETO isoform dependent mechanism that differentially regulates NETO/KAR trafficking in different cell types and subcellular compartments.

Vertebrate NETO1 and NETO2 have different C-terminal domains (Figure 4) that are required for the appropriate KAR targeting (Sheng et al., 2015). A phosphorylation site in the C-terminal domain of NETO2 (Lomash et al., 2017) could be involved in the NETO-isoform dependent differential trafficking of KAR subunits. It is also plausible that NETO1 can effectively compensate for the loss of NETO2 in GABAergic neurons, and therefore disguise the possible contribution of NETO2 in axonal and dendritic targeting of KAR subunits. Moreover, we cannot rule out the possibility that *Neto2* is insufficiently expressed in GAD67+ GABAergic neurons. Although our RT-PCR results show that cultured hippocampal neurons express both *Neto1* and *Neto2*, it does not resolve whether the GABAergic subpopulation that comprise 9% of the cultured neurons selectively expresses *Neto1*, but not *Neto2*.

GluK1c delivery in principal neurons was regulated by NETO1, but not NETO2 (Figure 5 B, C, D). Whereas GluK1b targeting in glutamatergic neurons is affected by both NETO1 and NETO2 (Figure 5 B, C, D). GluK1 splice variants GluK1c and GluK1b have different intracellular C-terminal

domains (Figure 1) (reviewed by Gonzalez-Gonzalez et al., 2012). GluK1c contains an extra sequence that could potentially mediate protein-protein interactions that might determine differential NETO-dependent regulation of GluK1 splice variants in principal neurons, where GluK1c is predominantly expressed (Vesikansa et al., 2012). Interestingly, axonal and dendritic targeting of both GluK1b and GluK1c depends on NETO1 in GABAergic neurons (Figure 5 E, F), suggesting that cell type dependent interactome could provide an indirect supplementary regulation mechanism for NETO/KAR targeting. For example, cell type specific regulation by covalent modifications could be achieved by the selection of kinases and phosphatases expressed in different cell types. Further studies are needed to resolve if KAR subunit interaction with NETOs could facilitate any of the numerous posttranslational modifications of KAR subunits including phosphorylation that promotes KAR membrane trafficking (Coussen, 2009; Gonzalez-Gonzalez et al., 2012).

GluK1 is highly expressed in the neonatal hippocampus and its' splice variant GluK1c is enriched in the principal cells, where its expression is proposed to underlie the immature-type tonic KAR activity (Vesikansa et al., 2012). NETO1-dependent axonal delivery of GluK1c could have physiologically significant role in regulating tonic presynaptic KAR activity at immature glutamatergic synapses in the hippocampus (Lauri et al., 2006; Vesikansa et al., 2012). Splice variant GluK1b expression is restricted to interneurons, where it could be incorporated to GluK1-containing immature-type tonic KAR activity implicated in regulating neuronal excitability in CA3 *stratum lucidum* interneurons (Segerstrale et al., 2010). It is possible that NETO1 is involved in regulating interneuronal t-KARs by affecting dendritic targeting of GluK1b. Combined, these data propose a cell-type specific role for NETO1 in physiologically significant KAR targeting mechanisms.

4.2 NETO/KAR complex in the formation of glutamatergic synapses in pyramidal cells and GABAergic interneurons (I, II, unpublished data)

4.2.1 The role of NETO/KAR complex in the differentiation of glutamatergic synapses in pyramidal neurons

KARs have been implicated to promote synaptogenesis both presynaptically and postsynaptically in the hippocampus (Tashiro et al., 2003; Ibarretxe et al., 2007; Vesikansa et al., 2007; Sakha et al., 2016). While NETOs have been established as important regulatory proteins of native and recombinant KARs, the role of NETOs in KAR-dependent synaptogenesis has not been studied before. Using cultured WT, Neto1KO and Neto2KO hippocampal neurons combined with presynaptic marker staining we found that Neto1KO MAP2 negative axons contained lower density Synaptophysin (Syn) puncta in comparison to WT or Neto2KO axons. Interestingly, the overexpression of GluK1c in Neto1KO neurons induced the Syn density to control level, thus effectively rescuing the impaired Syn density in the absence of NETO1. Other tested KAR subunits GluK1b and GluK2 did not have a similar Syn puncta promoting effect in the cultured Neto1KO neurons. As expected, Neto1KO dendrites also had a lower PSD95 puncta density suggesting a corresponding NETO1-associated impairment in postsynaptic differentiation (Figure 6 A) (Orav and Lauri, unpublished data). Interestingly, while there was no difference in the density, the area of PSD95 puncta were smaller in Neto2KO dendrites as compared to WT and Neto1KO (Figure 6 A) (Orav and Lauri, unpublished data). Smaller PSD95 puncta could indicate a developmental delay in

maturation of postsynaptic terminal or dendritic spines or both in the absence of NETO2 (Orav and Lauri, unpublished data).

These data suggest that NETO1 affects the presynaptic differentiation of cultured hippocampal pyramidal neurons likely by promoting GluK1c targeting to axons. NETO2, on the other hand, appears to participate in postsynaptically driven mechanisms involved in synaptic differentiation. This interpretation is supported by the findings of NETO-dependent KAR subunit targeting. NETO1, but not NETO2, affects the targeting of GluK1c – a presynaptic KAR subunit implicated in regulation of synaptic transmission and plasticity at CA3-CA1 (Lauri et al., 2006; Vesikansa et al., 2012). Therefore, NETO1-dependent axonal availability of GluK1c could induce the presynaptic

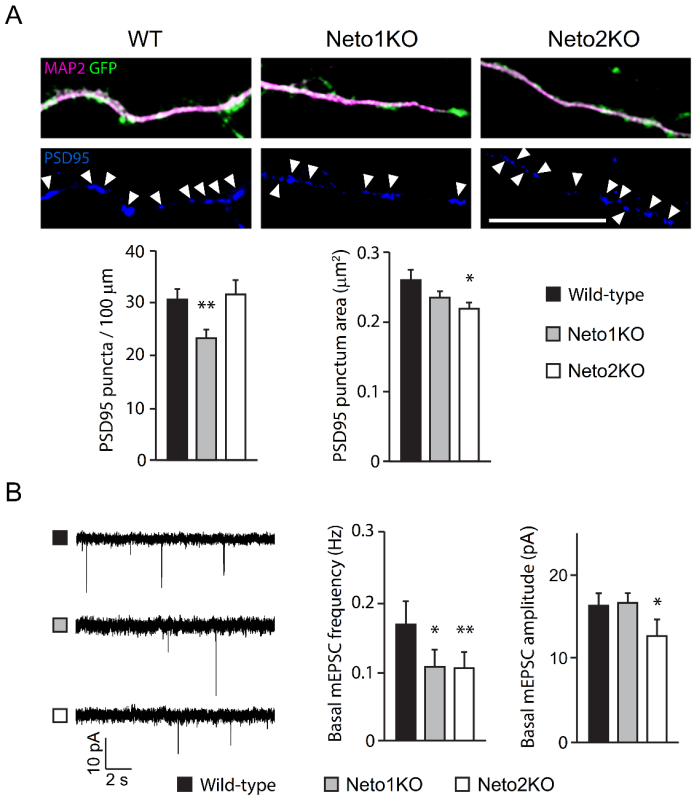


Figure 6. NETO2 regulates postsynaptic differentiation of glutamatergic CA1 synapses.

A. Example images of PSD95 puncta (blue, arrowheads) in the MAP2 positive (purple) dendrites of GFP infected (green) and quantified data on the mean density and area of PSD95 puncta in WT (n = 20, black bar), Neto1KO (n = 23, grey bar) and Neto2KO (n = 20, white bar) neurons. MAP2 and GFP signal is shown as merged. Scale bar 20 µm.
B. Example traces illustrating baseline mEPSC frequency and amplitude recorded from WT, Neto1KO and Neto2KO CA1 neurons of P4-P6 animals. Bar graphs present pooled data of baseline mEPSC frequency and amplitude in WT (n = 7, black), Neto1KO (n = 7, grey) and Neto2KO (n = 5, white).

differentiation of CA3-CA1. Indeed, GluK1c overexpression in Neto1KO neurons was able to rescue the presynaptic Synaptophysin puncta density to the WT control level.

To study the functional glutamatergic inputs in CA1 pyramidal neurons we recorded spontaneous action potential independent miniature excitatory postsynaptic currents (mEPSC) from WT, Neto1KO and Neto2KO acute hippocampal slices. We observed lower baseline mEPSC frequency in P4-P6 Neto1KO and Neto2KO, and lower baseline mEPSC amplitude in Neto2KO as compared to controls (Figure 6 B). As lower mEPSC frequency typically corresponds to lower number of presynaptic release sites, our electrophysiological data support the cell culture findings where the presynaptic puncta density was lower in the absence of NETO1, likely related to NETO1-dependent axonal targeting of GluK1c. NETO1/GluK1c role in the presynaptic differentiation of CA3-CA1 is further highlighted by a rescue experiment where GluK1c overexpression in CA3 pyramidal neurons of organotypic slices rescued the baseline mEPSC frequency to a WT control level.

Lower baseline mEPSC amplitude in Neto2KO CA1 neurons is in line with the postsynaptic phenotype observed in Neto2KO cultured glutamatergic neurons. Postsynaptic GluK2-containing KARs in CA3-CA1 synapse are implicated in synaptic expression of functional AMPARs and enhanced endosomal vesicle recycling in spines (Petrovic et al., 2017). Our findings suggest that the postsynaptic somatodendritic GluK2/5-containing KARs could be regulated by NETO2. We found that loss of NETO2 impaired the dendritic delivery of GluK2 and GluK5. Thus, it is possible that the postsynaptic differentiation of CA3-CA1 involves NETO2-dependent KAR subunits GluK2 and GluK5 that regulate spine maturation or AMPA receptor insertion to postsynaptic sites.

Taken together, our findings suggest a complementary role for NETO1 and NETO2 in the formation of glutamatergic CA3-CA1 synapse in the developing hippocampus. Axonal NETO1/GluK1c complex affects the presynaptic differentiation, while dendritic NETO2/GluK2 complex supports synapse maturation from a postsynaptic compartment.

4.2.2 The role of NETO1/KAR complex in glutamatergic synapse formation in GABAergic interneurons

KARs were recently found to regulate maturation of the dendritic tree in GABAergic interneurons (Jack et al., 2018), however, their role in synapse development has only been studied in glutamatergic cells. To identify GABAergic neurons in cell culture we used GAD67 labelling. Glutamatergic synapse cluster density was significantly lower in non-infected Neto1KO GAD67+ neurons as compared to controls. Overexpressing GluK1b or GluK2 rescued this phenotype in Neto1KO cultures to WT level. More detailed analysis of KAR and AMPA receptor containing synapses in GAD67+ neurons revealed synapse populations that contained AMPA receptors, KARs, and both. Interestingly, the density of GluA2/4 containing synapse clusters were not affected by the absence of NETO1 in GAD67+ cultured neurons. Whereas the KAR-containing synapse clusters were significantly reduced by the loss of NETO1.

Pharmacologically isolated AMPAR-KAR mediated mEPSC frequency and amplitude recorded from CA3 *stratum radiatum* interneurons was similar in WT and Neto1KO during the first week of postnatal development. Therefore AMPAR-containing glutamatergic inputs develop normally in the absence of NETO1, supporting the above described cell culture findings. Application of the GluK1-selective agonist ACET significantly reduced AMPAR-KAR mEPSC amplitude in P5 WT, but not

in Neto1KO interneurons. This result is consistent with NETO1-dependent loss of KAR-containing synapses in CA3 *stratum radiatum* interneurons.

NETO1 has been shown to affect NMDA receptors (NMDAR) by maintaining GluN2A at synapses (Ng et al., 2009) and by regulating NMDAR subunit composition (Wyeth et al., 2014). GluN2B is incorporated to NMDA receptors in the absence of NETO1 leading to increased GluN2B-selective antagonist Ifenprodil sensitivity of these receptors in Neto1KO mice (Wyeth et al., 2014). GluN2B subunit expression peaks during first postnatal week and declines during development (Petralia et al., 2005). Therefore we also studied NMDAR mediated mEPSCs and eEPSC, but found no significant differences in baseline mEPSC frequency, mEPSC amplitude, or Ifenprodil sensitivity of eEPSC amplitude between WT and Neto1KO CA3 *stratum radiatum* interneurons from P4-P6 mice. Thus, our findings suggest that NETO1 does not affect synaptic NMDAR during early postnatal development. However, it is possible that NETO1-dependent regulation of NMDAR composition becomes pronounced during later developmental stage when GluN2B is down regulated in WT (Wyeth et al., 2014).

4.2.3 Subunit and cell type dependent roles of NETO/KAR interaction in synaptogenesis

Our data indicates that NETO1 and NETO2 affect synaptogenesis by regulating KAR subunits in a subcellular compartment, synapse and cell type specific manner. In principal glutamatergic neurons, presynaptic expression of KAR subunits, in particular GluK1 and GluK2, have been shown to promote glutamatergic synapse formation (Sakha et al., 2016). Here we show that NETO1 is involved in GluK1c-dependent presynaptic differentiation of CA3-CA1 synapse by regulating GluK1c axonal delivery. While high-affinity KAR subunits GluK4 and GluK5 have not been found to induce formation of presynaptic puncta (Sakha et al., 2016), it has been proposed that GluK4 and GluK5 could promote synaptic localization of functional KARs without NETO1 and NETO2 (Palacios-Filardo et al., 2015). Indeed, we found that in the absence of NETO1 and NETO2 the axonal and dendritic targeting in glutamatergic neurons is reduced up to 50%, suggesting a NETO-independent mechanism partially involved in KAR trafficking. As high affinity KAR subunits cannot be expressed in the plasma membrane alone, they are likely incorporated in heteromeric KARs containing GluK1 and/or GluK2 subunits (reviewed by Gonzalez-Gonzalez et al., 2012).

According to our results, NETO2 is not involved in the presynaptic maturation. Instead NETO2 seems to have a complementary synaptogenesis promoting effect in the postsynaptic terminal. NETO2 could influence GluK2-mediated synaptic maturation (Petrovic et al., 2017) by regulating dendritic delivery of GluK2. As, in addition to KAR subunits, NETO2 interacts with KCC2 that has been shown to regulate actin dynamics and dendritic spine formation (Llano et al., 2015; Awad et al., 2018), we cannot rule out a KAR-independent mechanism in NETO2-dependent postsynaptic differentiation.

KAR-dependent regulation of excitatory synapse formation in GABAergic inhibitory neurons has not been studied before. A detailed analysis of glutamatergic synapses in GABAergic neurons revealed synapse subpopulations that contained AMPA receptors, KARs, and both. Interestingly, only KAR-containing synapse clusters were significantly reduced by the loss of NETO1. It has been previously suggested that NETO1 targets GluK1 selectively to AMPA-silent synapses in glutamatergic neurons (Sheng et al., 2015). Furthermore, KARs and AMPARs may be located at distinct synapse

populations in CA1 interneurons (Wondolowski & Frerking, 2009). However, here we show that in addition to two distinct KAR- and AMPAR-containing synapse populations, GABAergic neurons contain a third type of glutamatergic synapse subgroup where KAR subunits are incorporated to AMPAR-containing synapses. It is important to note that the KAR/AMPAR synapse population was observed in cultured neurons overexpressing KAR subunits that may be incorporated to subcellular compartments in a different manner as native KAR (Fièvre et al., 2016). Therefore, it remains to be confirmed if KAR/AMPAR-containing synapses in GABAergic neurons exist in native tissue.

Taken together, NETO1 in particular appears to have a dual role in glutamatergic synapse differentiation depending on the cell type. In pyramidal neurons, presynaptic NETO1-dependent KARs are directly involved in maturation of glutamatergic synapses and, consequently, AMPA receptor mediated synaptic transmission. However, in CA3 *stratum radiatum* interneurons, postsynaptic somatodendritic NETO1-dependent dendritic KARs affect the differentiation of strictly KAR-containing synapses that ultimately have a minor contribution to synaptic transmission. In contrast, NETO2 has a restricted role in KAR-dependent synaptogenesis, where NETO2-dependent postsynaptic differentiation of glutamatergic neurons could be achieved by regulating dendritic GluK2/5. NETO2 appears to not be involved in glutamatergic synapse formation in GABAergic neurons. To our knowledge this is the first description of NETO-dependent KAR mediated differentiation of glutamatergic synapses in both excitatory and inhibitory neurons of hippocampus.

4.3 Functional implications of NETO/KAR complex in principal cells and GABAergic interneurons in the immature hippocampus

4.3.1 Presynaptic NETO/KAR complex affects the maturation of CA3-CA1 synapse (I)

Tonically active presynaptic GluK1c-containing KARs in CA3-CA1 depress glutamate release, a function implicated in maturation of the emerging hippocampal circuitry (Lauri et al., 2006; Vesikansa et al., 2007). From our cell culture findings we concluded that NETO1 affects axonal presence of GluK1c. Therefore, we wondered if NETO1 could affect the activity of presynaptic GluK1-containing KARs present at CA3-CA1 synapse in the neonatal hippocampus (Lauri et al., 2006). Application of GluK1-selective agonist ACET (200 nM) increased mEPSC frequency in WT CA1 pyramidal neurons, but not in Neto1KO slices. In addition, frequency dependent facilitation of transmission at this synapse was not present in the absence of NETO1. Both these findings are consistent with the NETO1-dependent loss of presynaptic tonic KAR activity at immature CA3-CA1 synapses.

Using a potent GluK1-selective agonist ATPA on evoked synaptic responses we found that the attempt to strongly activate presynaptic GluK1-containing KARs did not lead to depression of transmission in Neto1KO during first postnatal week. This further confirmed that the loss of tonic KAR activity at immature CA3-CA1 synapses of Neto1KO mice is due to the absence of functional presynaptic GluK1-containing KARs rather than the reduced agonist affinity of these receptors. The significance of NETO1/GluK1c in the tonic KAR activity at CA3-CA1 was further emphasized by the finding that GluK1c overexpression in CA3 pyramidal neurons effectively rescued tonic presynaptic KAR activity in Neto1KO to WT level.

Finally, we explored NETO1/KAR complex in the functional development of CA3-CA1 at a network level. Microelectrode array recording of spontaneous network activity from cultured hippocampal slices revealed that firing of CA3 and CA1 pyramidal cells in Neto1KO is less synchronous as compared to wild-types. In addition, spike timing analysis suggested that in the absence of NETO1, connectivity between CA3 and CA1 remains weak as compared to WT. Importantly, the impaired development of synchrony and connectivity between CA3 and CA1 relies on the availability of GluK1c, as overexpressing GluK1c in CA3 pyramidal neurons rescued these defects in the functional development in Neto1KO slices to control level.

Taken together, the functional analysis uncovered the necessity of NETO1-dependent presynaptic GluK1c in the maturation of CA3-CA1 connectivity. Combined with the evidence from cell cultures, we propose that NETO1 regulates the axonal presence of GluK1-containing KARs that facilitate the formation and maturation of glutamatergic synapses connecting principal neurons in CA3 to CA1.

4.3.2 NETO1 affects ionotropic and metabotropic functions of somatodendritic KARs in CA3 stratum radiatum interneurons (II)

As discussed above, our cell culture experiments indicated that dendritic and axonal targeting of KAR subunits GluK1 and GluK2 in GAD67+ GABAergic neurons depends on NETO1, but not NETO2. In addition, our data suggested that NETO1/KAR complex is involved in the formation of KAR-containing synapses in GABAergic cultured neurons. To understand the functional implications of NETO1/KAR complex in the immature GABAergic neurons, we performed electrophysiological recordings from CA3 *stratum radiatum* interneurons in acute hippocampal slices from neonatal WT and Neto1KO mice.

Postsynaptic KARs contribute to synaptic transmission at certain interneurons (Cossart et al., 1998; Frerking et al. 1998; Clarke et al., 2012). We showed that postsynaptic KARs are present already during first week after birth in WT CA3 *stratum radiatum* interneurons where they mediated modest postsynaptic current ($EPSC_{KA}$) that is not observed in Neto1KO slices. Thus, these findings for the first time describe the presence of NETO1-dependent postsynaptic KARs at immature CA3 *stratum radiatum* interneurons. Combined with cell culture findings, it is likely that the ionotropic NETO1-dependent KARs operate only in a subpopulation of glutamatergic synapses and therefore exhibit a modest contribution to overall synaptic transmission during the early postnatal development.

In addition to the ionotropic function, somatodendritic KARs in CA3 interneurons have a metabotropic G-protein coupled mode-of-action that inhibits I_{mAHP} during the first postnatal week (Segerstrale et al., 2010). In line with the published findings, we observed GluK1-dependent inhibition of I_{mAHP} in a subpopulation of CA3 *stratum radiatum* interneurons. In contrast to WT, I_{mAHP} amplitude was non-responsive to GluK1-selective agonist ATPA in Neto1KO interneurons located in CA3 *stratum radiatum* suggesting impaired metabotropic KAR signaling in the absence of NETO1.

Interestingly, ATPA application induced an inward current in Neto1KO interneurons albeit significantly smaller as compared to WT. It is likely that a subpopulation of ionotropic KARs are trafficked to plasma membrane in the absence of NETO1. Our cell culture data show that the dendritic delivery of all tested KAR subunits is reduced to ca 75% in the absence of NETO1 in GABAergic neurons. Thus, it is possible that NETO1-independent mechanisms exist that additionally regulate dendritic targeting of KARs in GABAergic neurons. For instance in recombinant system, it has been shown that high-affinity KAR subunits GluK4 and GluK5 can promote the surface expression and

synaptic localization of low affinity KAR subunits GluK1 and GluK2 in the absence of NETO1 and NETO2 (Palacios-Filardo et al., 2015). This could be a plausible mechanism for NETO1-independent KAR trafficking in GABAergic neurons, as they have been shown to endogenously express high-affinity GluK5 (Wyeth et al., 2014). However, we cannot rule out the possibility that in the absence of NETO1 some GluK1-containing KARs are retained in the plasma membrane of the neuron cell body, where they can elicit an inward current albeit smaller as compared to WT.

Together, these findings demonstrate that NETO1 affects the postsynaptic and metabotropic KAR signaling likely by regulating dendritic delivery of KAR subunits in CA3 *stratum radiatum* interneurons. However, a subpopulation of ionotropic GluK1 subunit containing KARs that probably do not localize to postsynaptic site remain functional in the absence of NETO1.

4.3.3 NETO1/KAR complex has no significant effect on interneuron excitability and early network activity in immature hippocampus (II)

As most, but not all, KAR functions become impaired in Neto1-null immature CA3 *stratum radiatum* interneurons, we went on to study interneuron excitability and early network activity that have been shown to be modulated by hippocampal KARs (Lauri et al., 2005; Segerstrale et al., 2010; Juuri et al., 2010). Unexpectedly, loss of NETO1 did not affect interneuron excitability during the first postnatal week, however, by P10 spontaneous action potential firing was lower in Neto1KO.

Early network activity that is characterized by spontaneously occurring network bursts (Ben-Ari, 2001) was not affected by NETO1 deficiency. The basal frequency of network bursts, basal frequency of spontaneous inhibitory postsynaptic current (sIPSC) and basal amplitude of spontaneous excitatory postsynaptic currents (sEPSC) recorded from CA3 pyramidal neurons were not different between WT and Neto1KO. Only basal amplitude of sIPSC and basal frequency of sEPSC were lower in the absence of NETO1. While loss of NETO1 did not have a dramatic phenotype regarding the baseline network activity, Neto1KO network was less sensitive to Kainate. Low concentration of Kainate robustly increased the frequency of network bursts as well as sIPSC frequency and sEPSC amplitude in WT. Kainate application did not affect sIPSC frequency and sEPSC amplitude in Neto1KO as compared to WT. Kainate treatment slightly increased the network burst frequency in Neto1KO, but it was significantly smaller as compared to a robust increase in WT.

Thus, KAR interaction with NETO1 in CA3 interneurons did not affect basal neuron excitability and did not have a direct effect on the early network bursts during first week of postnatal development. The excitability of the neonatal network is under strict homeostatic control (Huupponen et al., 2007; Colin LeBrun et al., 2004; Desai et al., 2002; reviewed by Tien and Kerschensteiner, 2018), therefore any NETO1-dependent mechanisms regulating action potential firing frequency could be compensated in a knock-out model.

Taken together, NETO-dependent delivery of KAR subunits is instrumental in the hippocampal circuit maturation. In particular, NETO1-dependent GluK1-containing presynaptic KARs in CA3 neurons induce maturation of functional connectivity between CA3 and CA1. Interneuronal somatodendritic KARs promote GluK-containing synapse formation, mediate KAR signaling and modulate network activity in NETO1-dependent manner. Whereas NETO2-dependent synapse maturation phenotype is restricted to postsynaptic compartment of glutamatergic CA3-CA1 synapse.

5. CONCLUSIONS

Kainate-type glutamate receptors modulate synaptic transmission in adult brain as well as during brain development. NETO proteins were identified as auxiliary subunits for KARs originally in 2009 (Zhang et al., 2009; Tang et al., 2011), however, our knowledge on their physiological significance in the brain is still limited. In particular, very little is known on the functions of NETOs in the developing brain where KARs are widely expressed. Therefore, in this study we addressed NETO-dependent regulation of KARs at different synapses in pyramidal cells as well as interneurons in the developing hippocampus. The main novel findings of the thesis are the following

I) We found that in principal neurons both NETO1 and NETO2 promote axonal and dendritic targeting of most KAR subunits, with the interesting exception of GluK1c subunit that was selectively regulated by NETO1. In contrast, only NETO1 is involved in regulating the distal delivery of KAR subunits in GAD67+ GABAergic neurons, in a subunit independent manner. NETO-dependent differential axonal and dendritic delivery of KAR subunits underlies KAR functions in the different subcellular compartments and cell types and consequently affects the KAR dependent synaptic differentiation and circuit maturation in hippocampus.

II) In principal neurons, GluK1c-driven presynaptic differentiation depends selectively on NETO1, whereas NETO2-dependent dendritic GluK2 could be involved in postsynaptic differentiation. In GABAergic neurons, dendritic NETO1 facilitates formation of glutamatergic KAR-containing synapses, while AMPA-containing synapse development is apparently not affected by NETO1. NETO1 regulates the KAR-containing synapse formation by promoting dendritic delivery of KAR subunits in GABAergic interneurons.

III) NETO1-dependent axonal GluK1 is required for physiologically important tonic presynaptic KAR activity at immature CA3-CA1 synapse. Loss of presynaptic KAR activity in the absence of NETO1 leads to functional decoupling of areas CA3 and CA1 that is effectively rescued by GluK1c overexpression in CA3 pyramidal neurons.

IV) In CA3 *stratum radiatum* interneurons, loss of NETO1 significantly impairs both ionotropic and metabotropic signaling of KARs. Nevertheless, CA3 interneurons contain a subpopulation of somatodendritic KARs that remain functional in the absence of NETO1. NETO1 is central for KA-dependent modulation of the network activity and GABAergic synaptic transmission during early development. Although NETO1 is required for many of the functions ascribed to KARs in immature CA3 interneurons, these functions are not indispensable as NETO1 deficiency does not have severe consequences on the excitability of the CA3 network during early postnatal development.

Combined, our findings highlight the cell type dependent differences in NETO-driven regulation of KARs in the immature hippocampus. We show a previously undescribed NETO-dependent regulation of presynaptic KARs that are important for maturation of the glutamatergic synapses. Further, we provide first data on NETO1-dependent regulation of KAR subunit targeting and formation of KAR-containing synapses at GABAergic neurons. Thus, by regulating both pre- and postsynaptic KARs in pyramidal cells and interneurons in immature hippocampus, NETO1 is an important modulator of hippocampal circuit development. In principal neurons, NETO1-dependent loss of GluK1c signaling

leads to impaired glutamatergic synapse maturation and consequently delayed development of functional connectivity between CA3 and CA1. Whereas, in GABAergic neurons, loss of NETO1 results in impaired KAR-containing synapse differentiation, however with minor consequences at the network level.

Unraveling the precise molecular mechanisms regulating immature KAR activity is critical to elucidate physiological maturation of hippocampal circuits. In addition, detailed knowledge of mechanisms governing immature-type KARs could be instrumental in understanding developmentally originating neurological disease pathogenesis as well as in developing appropriate therapeutic strategies.

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